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Isolation of the human PC6 gene encoding the putative host protease for HIV-1 gp160 processing in CD4$^+$ T lymphocytes

(HIV/AIDS/Kex2-like proteases/T cells)

LUIS MIRANDA*†, JOSEPH WOLF*†‡, SERGIO PICHUANTES§, RICHARD DUKE¶, AND ALEX FRANZUSOFF*‖

*Department of Cellular and Structural Biology, Molecular Biology Program, and ¶Medical Oncology, University of Colorado Cancer Center, University of Colorado Health Sciences Center, Box B-111, 4200 East 9th Avenue, Denver, CO 80262; and §Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608

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ABSTRACT **Production of infectious HIV-1 virions is dependent on the processing of envelope glycoprotein gp160 by a host cell protease. The protease in human CD4$^+$ T lymphocytes has not been unequivocally identified, yet members of the family of mammalian subtilisin-like protein convertases (SPCs), which are soluble or membrane-bound proteases of the secretory pathway, best fulfill the criteria. These proteases are required for proprotein maturation and cleave at paired basic amino acid motifs in numerous cellular and viral glycoprotein precursors, both *in vivo* and *in vitro*. To identify the gp160 processing protease, we have used reverse transcription-PCR and Northern blot analyses to ascertain the spectrum of SPC proteases in human CD4$^+$ T cells. We have cloned novel members of the SPC family, known as the human PC6 genes. Two isoforms of the hPC6 protease are expressed in human T cells, hPC6A and the larger hPC6B. The patterns of SPC gene expression in human T cells has been compared with the furin-defective LoVo cell line, both of which are competent in the production of infectious HIV virions. This comparison led to the conclusion that the hPC6 gene products are the most likely candidates for the host cell protease responsible for HIV-1 gp160 processing in human CD4$^+$ T cells.**

Critical steps in HIV infection include the recognition, binding, and fusion with CD4$^+$ cells—e.g., T cells and macrophages. Contact between HIV virions and human T cells is mediated by subunits of the envelope glycoprotein gp160 on the viral surface with the T cell CD4 protein. Mature HIV-1 envelope glycoproteins consist of two subunits: gp120, which binds to the CD4 protein; and the transmembrane gp41 protein, which promotes fusion of the viral envelope and delivery of the nucleocapsid. These two subunits are derived from the HIV-1 gp160 precursor polypeptide while in transit through the host cell secretory pathway (1–3). In fact, the spread of HIV can be mediated through the assembly and release of new virions at the plasma membrane of infected cells, or by direct transmission through the fusion of infected cells with uninfected CD4$^+$ cells, giving rise to multi-nucleated syncytia (3, 4–7). Either scenario contributes to depletion of the functional T-cell population in HIV-infected patients. Consequently, proper processing of the HIV surface glycoproteins is critical to viral infectivity and to the collapse of the immune system in AIDS.

The cleavage junction in gp160 consists of a highly conserved sequence, Arg-X-Lys/Arg-Arg (with paired basic amino acid motif underlined), also found in the glycoproteins of other enveloped viruses, as well as in cellular precursor proteins (8–10). Site-directed mutagenesis of amino acids at this junction eliminated gp160 cleavage by the cellular protease (6, 11–15). The loss of gp160 processing resulted in the incorporation of the inactive gp160 precursor into virions, giving rise to uninfectious virions, and to HIV-infected cells that were unable to form multinucleated syncytia.

Polypeptide cleavage at dibasic amino acid motifs has been relegated to members of an emerging family of endoproteases, the subtilisin-like protein convertases, or SPCs. This nomenclature stems from conservation of the protease catalytic mechanism with that of the bacterial subtilisins. The archetypal eukaryotic member of this family is the Kex2 protease in the yeast *Saccharomyces cerevisiae* (16–18). Based on homology with yeast *KEX2*, a number of mammalian SPC genes were isolated, including furin (19, 20), PC1/PC3 (21–23), PC2 (22, 24), PC4 (25, 26), PACE4 (27, 28), PC5/PC6A (29, 30), PC6B (31), and PC7 (32). Furin and PACE4 exhibit broad tissue and organ expression patterns by Northern blot analysis. These proteases apparently process a variety of cellular proteins transiting the "constitutive" secretory pathway. PC1 and PC2 display a more limited expression pattern in endocrine and neuroendocrine cells, with a role in hormone processing within the "regulated" secretory pathway of those cell types.

We have shown that heterologous expression of HIV-1 gp160 in yeast resulted in the proper targeting and processing of the protein in the secretory pathway (33). Using genetic and biochemical approaches, the yeast Kex2 protease was shown to be both necessary and sufficient for correctly processing gp160 (33, 34). That work supported other studies implicating a mammalian member(s) of the SPC protease family in HIV-1 gp160 processing. In the work presented here, RT-PCR and Northern blot analyses were used to ascertain which SPC protease is available in human CD4$^+$ T cells for HIV-1 gp160 processing.

MATERIALS AND METHODS

Cell Lines. CEM T-cell, Jurkat T-cell, and LoVo human colon adenocarcinoma cell lines were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and glutamine. Fresh blood from a healthy adult donor was used to isolate CD4$^+$ cells using MicroCELLector T-25 cell culture flasks (Applied Immune Sciences, Santa Clara, CA). Purity of the CD4$^+$ population was verified by FACS analysis on an EPICS C FACS analyzer (Coulter) using rhodamine-conjugated anti-human CD4 antibody.

RNA Isolation. Total RNA was isolated using Trizol reagent (GIBCO/BRL) from cultured cells harvested in log growth phase, from isolated CD4$^+$ T cells, or from BALB/c mouse brain that was directly homogenized in Trizol reagent. Poly(A)$^+$ RNA

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Abbreviations: SPC, subtilisin-like protein convertases; RT, reverse transcription; HA, hemagglutinin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U56387).

†L.M. and J.W. contributed equally to this work.

‡Present address: Peace College, 15 East Peace Street, Raleigh, NC 27604.

‖To whom reprint requests should be addressed. e-mail: Alex.Franzusoff@UCHSC.Edu.

was isolated using the PolyATtract mRNA Isolation System (Promega).

Reverse Transcription (RT)-PCR Analysis. Degenerate oligonucleotide primers were designed corresponding to the consensus peptide sequences from the catalytic domain of all known mammalian SPC proteases. First strand cDNA synthesis was performed using 1 μg of total RNA from CEM cells in the presence of Superscript II reverse transcriptase (GIBCO/BRL) and the antisense primer 5′-HYCCCABSWRTGRRYDGWCATGAA-3′. The sense primers 5′- TGYGGNGTHGGHGTDGCHTA-YAAYKCC-3′, 5′-GGSWSCATCTWYGTSTGGGCYTC-3′, and the antisense primers 5′-CAGRTGYTGCATRTCYCKC-CAKGTVAG-3′ and 5′- SGGDGCWGMDGCHGAKGTSC-CHGWRTG-3′ were used interchangeably in PCR reactions to obtain cDNA fragments of the SPC proteases. Reaction mixtures for PCR contained cDNA template, 100 pmol each of the degenerate primers, and 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus) in 10 mM Tris·HCl (pH 8.3), 50 mM KCl, and 2 mM $MgCl_2$ containing a mixture of dNTPs (0.2 mM each). Reactions were carried out in a thermocycler (Coy Laboratory Products, Ann Arbor, MI) for 30 cycles of denaturation (94°C for 1 min), annealing (52°C for 1 min), and extension (72°C for 2 min). After resolution on 1% low-melting agarose gels (FMC), the PCR products were purified by agarase treatment (New England Biolabs) and subcloned into pCR-Script vector (Stratagene).

Northern and Southern Blot Analyses. Northern and Southern blots were performed essentially according to Sambrook *et al.* (35). Radiolabeled DNA probes were generated by the Radprime DNA labeling system (GIBCO/BRL) in the presence of α^{32}P-dATP (Amersham). The catalytic domain probe used in the Southern and Northern blot analyses was derived from the hPC6A gene, using nucleotides 439-1210 described in Fig. 2. The PC6-specific probe was generated from DNA at the 5′ end of the hPC6A open reading frame (nucleotides 16–416, Fig. 2). Radiolabeled DNA probes for the human furin, PACE4, and PC1 blots were derived from unique sequences downstream of the catalytic domain. The human PC1 DNA probe used in Fig. 3 and the mouse gene sequence are ≈91% identical.

Molecular Cloning and Sequencing of the Full-Length Human PC6A cDNA. RT-PCR from 0.5 μg of CEM poly(A)$^+$ RNA was performed as described above, except that a longer extension time was required and *Taq* polymerase was mixed with Vent (exo$^+$) DNA polymerase (New England Biolabs) at a ratio of 40:1 to ensure fidelity (36). A 2.4-kb PCR product corresponding to about 86% of the coding sequence of hPC6 was obtained by using the degenerate sense primer 5′-CCAAGYATGTGGTAYATG-CAYTGYAGY-3′ and the antisense primer 5′-GGCTGCT-CAGCCTTGGAATGTACATGTTTT-3′. A 0.5-kb fragment corresponding to the remaining coding sequence at the 5′ end of hPC6 was obtained using the degenerate sense primer 5′-AGCGTNGGNACNATGGAYTGGGAYTGG-3′ and antisense primer 5′- RTTRTCRCTRCARTGCATRTACCACAT-3′: H = A, T, or C; Y = C or T; B = G, T, or C; S = G or C; W = A or T; R = A or G; D = G, A, or T; N = G, A, T, or C; K = G or T; V = G, A, or C. These primers correspond to nucleotides 2755–2784, 430–456, 19–45, and 436–462, respectively, of the mouse/rat PC6 sequence (29). PCR gene splicing by overlap extension (37) was carried out to join both products and form the full-length hPC6. Cloned cDNAs were sequenced in both directions using M13 forward, reverse, and specific 5′ end-labeled fluorescent primers on a Pharmacia automatic ALF DNA sequencer.

RESULTS

RT-PCR Cloning of SPC cDNAs from a Human T-Cell Line. The eukaryotic SPC gene family is defined by the catalytic mechanism of these serine proteases. The domain of amino acids involving the protease active site is known as the catalytic domain, and is highly conserved among all the members of the SPC family (8, 9). To identify the members of the SPC gene family present in human CD4$^+$ T lymphocytes, RT-PCR was performed using degenerate primers of the catalytic domain. For this preliminary evaluation, a human CEM (CD4$^+$ T lymphocyte) cell line was used as the source of RNA because of its effectiveness as host cells for *in vitro* HIV infection and viral production, (e.g., ref. 38).

The PCR reaction yielded a single band of expected size by agarose gel electrophoresis, presumably representing a heterogenous mixture of the different SPC mRNAs present in the CEM T cells. To determine their identity, DNA sequencing of several clones made from the PCR band was done, yielding two distinct sequences. Comparison with the GenBank sequence database revealed that one class of cDNAs encoded human furin (GenBank accession number X17094). Although human furin had been cloned and its expression in T cells has been described (20, 39), the furin clones in our RT-PCR products validated our strategy for identifying SPC genes. The other class of cDNA clones encoded a novel human protein, with highest similarity to mouse/rat PC6 (95.1% nucleotide identity, 85% amino acid identity, Fig. 1*B*). The catalytic domain sequence from this new clone, hPC6, was also compared with other known human SPC enzymes PACE4, furin, PC1, and PC2, as shown in Fig. 1*B*. By this comparison, the furin catalytic domain exhibited the highest nucleotide identity to hPC6, whereas the PACE4 catalytic domain shared the highest amino acid identity with hPC6. The three active site residues characteristic of the serine protease class (Asp, His, and Ser) are found in similar positions to their SPC family counterparts.

Cloning and Analysis of the Human PC6A Gene. To clone the full-length human PC6A cDNA, RT-PCR reactions with RNA from the human CEM T-cell line were performed again with primers designed from the rat/mouse PC6A sequences 5′ and 3′

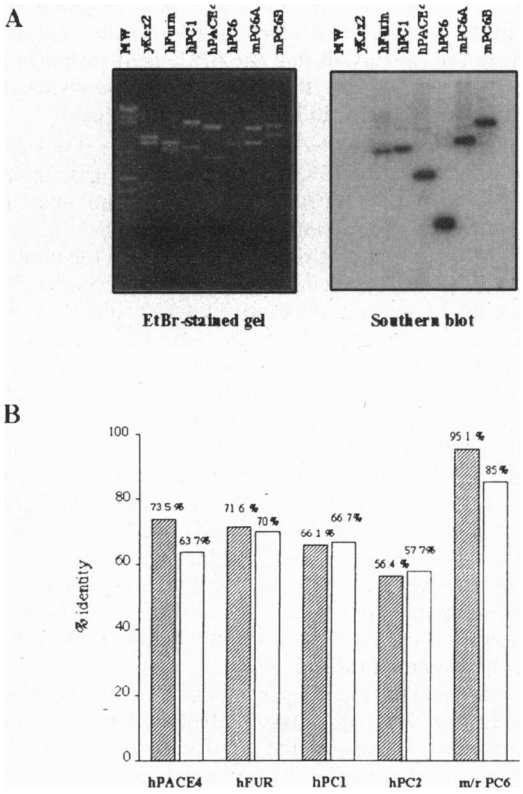


FIG. 1. Conservation of catalytic domain sequences between members of the mammalian SPC family. (*A*) Southern blot analysis of cloned SPC gene family members. Designations for organisms are y (yeast), h (human), and m (mouse). (*Left*) Ethidium bromide stained gel. (*Right*) DNA bands detected upon hybridization with a ^{32}P-labeled probe from the catalytic domain of the cloned human PC6 gene. MW are DNA marker bands. (*B*) Percent identity between hPC6A catalytic domain and SPC family members.

to the catalytic domain. Recombinant PCR was done to assemble the full-length gene. The complete hPC6A cDNA and predicted amino acid sequences are presented in Fig. 2. The similarity of the human PC6A to the rat/mouse homologues is striking: ≈90% at the nucleotide level and 96% at the amino acid level (29, 30). The hPC6A gene is predicted to encode a 915-residue precursor polypeptide with a 34-amino acid signal peptide for targeting to the endoplasmic reticulum and six potential *N*-linked glycosylation sites. The C-terminal domain of hPC6A is cysteine-rich with 56 cysteines, similar to the cysteine-rich regions found in other SPC proteases. The role of the cysteine-rich domain in SPC enzymes is being studied in a number of laboratories. Although these enzymes generally show requirements for binding calcium ions near the active site for protease activity, other divalent ions, such as copper and zinc, are potent inhibitors of SPC protease activity (9, 40). Hence, the cysteine-rich domain may function in regulating metal ion binding and protein folding for catalytic activity in the appropriate subcellular compartments where these proteases function.

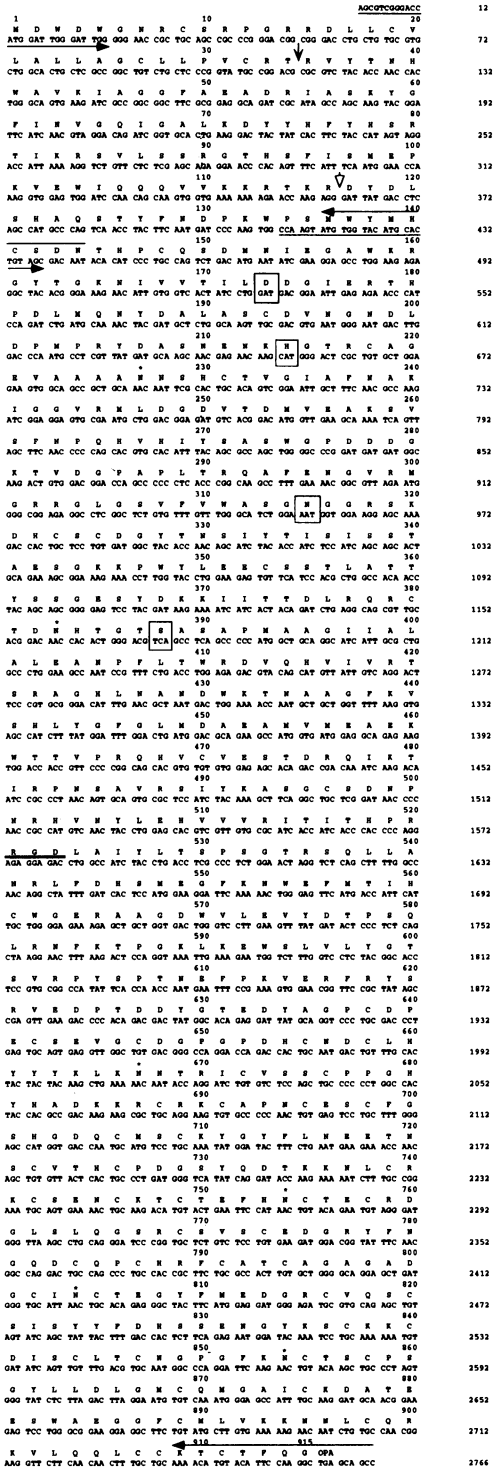
The hPC6 Catalytic Domain Probe Detects Other Members of the SPC Gene Family. The results of the RT-PCR reaction suggested that hFurin and hPC6 were expressed in human CD4$^+$ T cells. To test whether the RT-PCR approach had revealed the entire spectrum of SPC enzymes in human CD4$^+$ T cells, Northern blot analysis was performed on RNA isolated from a variety of cell lines and primary T lymphocytes. To ensure that the probe used for the Northern blots was broadly specific for all different members of the mammalian SPC family, Southern DNA hybridization was evaluated with several previously cloned SPC genes [excluding PC2 and PC4, which show only testis- and/or neuroendocrine-specific expression (10)]. The hPC6 catalytic domain was a logical choice for such a probe because of its high degree of similarity to the other members of this gene family (Fig. 1*B*). As shown in Fig. 1*A*, the hPC6 catalytic domain probe hybridized strongly to the mammalian SPC genes. Detection of the yeast *KEX2* gene was evident, though weaker, reflecting less sequence identity (51%) between the yeast and human genes (41). These results support the use of the hPC6A catalytic domain probe in Northern blot analysis to assess the presence of all SPC mRNAs in human CD4$^+$ T cells.

Northern RNA Analysis of SPC Expression in Human CD4$^+$ CEM Cells. To test whether hPC6 and hFurin were the only SPC genes expressed in the CEM human T cell line, Northern hybridization analysis was performed using the hPC6 catalytic domain probe. A pattern of three bands was observed (Fig. 3*A*, lane 1). The fainter central band represented hFurin, as shown by furin-specific probes (Fig. 3*A*, lane 4). Neither PACE4 nor PC1 were detected in human CEM cells (Fig. 3*A*, lanes 7 and 10).

The other two hybridizing RNA bands found in human CEM cells were two isoforms of PC6 originally detected in mouse cells: PC6A (3.5 kb) and PC6B (6.5 kb) (31). Although cloning and sequencing of the 0.4-kb 5′ end of the mouse PC6B gene has not been reported, the nucleotide sequence of the two PC6 isoforms was identical over 2.3 kb of the remaining 2.4 kb length of PC6A (31). Mouse PC6B is longer than PC6A, together with a putative membrane-spanning and cytosolic tail domains. Hence, the results from the Northern blot analysis reiterated the RT-PCR analysis that CEM human T cells only expressed furin and PC6 members of the SPC family.

Comparison of SPC Gene Expression in Human CD4$^+$ T Cells and HIV-Competent LoVo Colon Cells. To rule out possible artifactual contributions of cultured T-lymphocyte cell lines to our assessment of SPC gene expression, we evaluated the pattern of PC6 gene expression in the Jurkat T cell line, which is competent for HIV-1 virion production, and in primary CD4$^+$ T lymphocytes. Primary CD4$^+$ T lymphocytes were purified from peripheral blood of a healthy adult donor by the panning technique and assayed for enrichment by fluorescence activated cell sorting (FACS) analysis. By this method, CD4$^+$ T lymphocytes comprised ≈85% of the enriched cell population (not shown).

A



B

Convertases	hPC6A	hPC1	hPC2	hfurin
hPC1	63%			
hPC2	57%	54%		
hfurin	65%	62%	56%	
hPACE4	69%	62%	51%	68%

FIG. 2. Nucleotide and deduced amino acid sequence of the cDNA-encoding human PC6A. (*A*) The catalytic amino acids Asp, His, Ser, and the important Asn are boxed. Putative cleavage site of the signal peptide and propeptide are indicated by a vertical arrowhead and arrow, respectively. Potential *N*-glycosylation sites are indicated by an asterisk. The Arg-Gly-Asp sequence is underlined. Horizontal arrows along the sequence illustrate the oligonucleotides used in the cloning. (*B*) Amino acid identity among full-length human SPC enzymes.

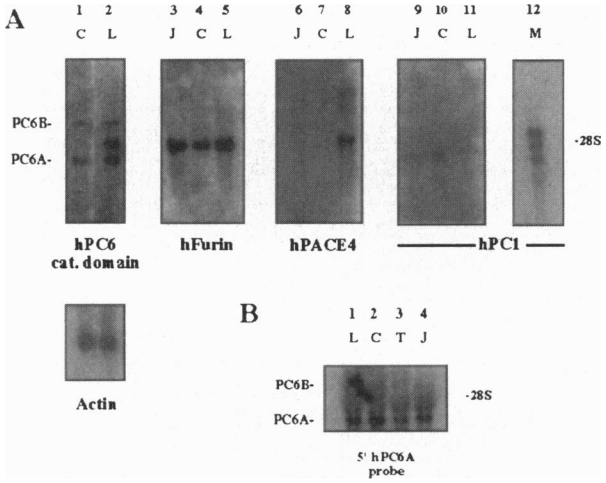


FIG. 3. Northern blot analyses of SPC expression in primary CD4+ T cells, LoVo, and T-cell lines. (*A*) Northern blot analysis of ≈1 μg of mRNA (lanes 1 and 2) or ≈10 μg of total RNA (lanes 3–12) from Jurkat (J), CEM (C), and LoVo (L) cells, and mouse brain (M) with probes made from hPC6A catalytic domain, other SPC genes or actin, as noted. 28S indicates rRNA mobility. (*B*) Northern blot analysis of total RNA from Jurkat (J), CEM (C), and LoVo (L) cells, or from freshly isolated primary CD4+ T lymphocytes (T) was performed using a probe made from unique sequences at the 5′ end of hPC6A.

Northern hybridization was performed on total RNA from CEM and Jurkat T cell lines, and from freshly isolated primary human CD4+ T cells. An hPC6-specific probe derived from the 5′ end of the cDNA was used for hybridization (Fig. 3*B*), together with an actin-specific probe to ensure equal loading and RNA integrity (data not shown). PC6A mRNA was detected to the same level in all of the different CD4+ T cells tested. Interestingly, PC6B mRNA was not efficiently detected with this 5′ hPC6 probe, perhaps reflecting sequence differences between PC6B and PC6A at the 5′ end (31). We are currently cloning the full-length hPC6B cDNA to resolve this point. However, the results from this Northern blot analysis indicated that detection of PC6 in human T cells was not an artifact of tissue culture growth conditions.

LoVo cells, derived from a human colon adenocarcinoma (42), have proven extremely valuable for testing the efficiency of cleavage of a variety of transfected-precursor proteins by the SPC proteases. LoVo cells express full-length furin mRNA, yet the sequence in LoVo cells encodes a truncated, defective furin protease (43). To that end, processing of Newcastle disease virus envelope F_o glycoprotein, the avian influenza hemagglutinin (HA) protein, diphtheria toxin, hepatocyte growth factor/scatter factor receptor, and transforming growth factor-β were all eliminated when expressed in LoVo cells, unless a wild-type furin gene was introduced as well (44–48). Unexpectedly, Ohnishi *et al.* (47) demonstrated that transfection of the HIV-1 genome into LoVo cells gave rise to infectious virions, and that the HIV-1 gp160 precursor processing was unaffected by the absence of furin in those cells.

Because LoVo cells do not depend on furin for HIV-1 gp160 processing, we examined the cohort of SPC enzymes expressed in human LoVo cells. Northern RNA analyses were performed with the same probes as for human T cells (Fig. 3). The pattern of SPC expression in LoVo cells was slightly more complex than in T cells. Although only three bands were evident by probing with the catalytic domain probe (Fig. 3*A*, lane 2), the central band was more pronounced in LoVo cells than in human T cells. Probing with specific domains of the SPC enzymes revealed that hFurin was expressed, as expected (lane 5). In addition, hPACE4 (lane 8) was represented in the central band of RNA from LoVo cells, but not from human T cells. In contrast, hPC1 was not detected in either LoVo cells (Fig. 3*A*, lane 11) or human T cells (lane 10). Surprisingly, both PC6 isoforms were evident in LoVo cells as in human T cells (Fig. 3 *A*, lane 2, and *B*, lane 1). These results indicate that the only differences in the SPC pattern of LoVo versus human T cells were the expression of hPACE4 and of a mutant furin gene product.

DISCUSSION

In this paper we have identified the human PC6 genes, hPC6A and hPC6B, in CD4+ T lymphocytes and LoVo cells. The human PC6 gene products represent new members of the Kex2-like, SPC family, whose catalytic mechanism belongs to the serine protease class (9). The cDNA encoding the full-length hPC6A was cloned from human CEM cells, and its sequence was determined. The human and rat/mouse genes share 96% amino acid identity (29–31). Although the 5′ end of the mouse PC6B (mPC6B) has not been cloned, the two mPC6 isoforms share sequence identity from amino acid 330 to amino acid 877 (31). Based on apparent sequence motif similarities with other SPC enzymes, cleavage of the hPC6A protease pro-region is predicted following the dibasic amino acids Lys-Arg^{117} (9, 10, 29, 49). This zymogen processing event would yield a 799-amino acid hPC6A protease. While furin and PC1 are suggested to undergo autocatalytic processing of the zymogen to the mature protease prior to exit from the endoplasmic reticulum (49–53), PC2 is bound to a chaperone protein, e.g., 7B2 in neuroendocrine cells, and is only processed to the mature size in the distal Golgi compartments (54, 55). SPC enzymes typically display functional protease activity upon reaching the distal Golgi compartments, where presumably the more acidic pH conditions promote the final transition to an active conformation (56). This final transition may represent dissociation of the noncovalently bound pro-segment or the chaperone protein from the mature protease.

The PC6A protease apparently functions as a soluble enzyme within the secretory pathway. The longer mPC6B gene encodes a 190-kDa type I membrane protein with an extended cysteine-rich domain, a single transmembrane region and an 87-residue cytosolic domain (31). Subcellular localization studies have not been described for the PC6 proteases, so it is not yet clear where they reside or cleave their precursor substrates. These issues are relevant with regards to processing of precursors within the constitutive versus regulated secretory pathways. The naturally low abundance of the SPC proteases within the cell has hampered unequivocal resolution of these questions.

Data on the distribution of PC6 in mouse organs shows expression in intestine and adrenal glands more abundantly than in lung, ovaries, and testis, with less perceptible expression in brain, spleen, kidney, and liver (10, 29, 30). In those studies, not all organs or cell types expressing PC6 exhibited similar levels of both isoforms, suggesting that their expression is not coordinately linked.

Some experiments have been performed with the mouse PC6A enzyme to define the substrate specificity of this protease both in *vitro* and in *vivo*. Coexpression of mPC6A with native or mutant pro-renin showed that the processing efficiency at dibasic amino acid motifs is improved by the presence of Arg at the P4 or P6 position upstream of the cleavage junction (46). In the case of furin, the upstream basic amino acid is *obligatory* for processing (46, 57–59).

Experiments to test *in vivo* activity of the SPC proteases by overexpression studies provide valuable information, yet should be interpreted with caution. For instance, efforts to test the role of mPC6 and other SPC proteases in influenza HA precursor processing have been performed (46, 60, 61). HA protein, when introduced into a CV-1 kidney fibroblast cell line, showed ≈86% efficiency of processing by the endogenous protease (46). On the other hand, in the furin-defective LoVo cells, HA precursor processing was undetectable. Overexpression in LoVo cells of either PC6 or furin, but not PACE4, restored efficient processing of HA precursors. These results indicate that both PC6 and furin are competent to cleave the substrate *in situ*, yet creates ambiguity for interpretation about the bona fide protease. Protease over-

expression clearly overwhelms mechanisms for retaining these proteases in their proper subcellular compartment, leading to inappropriate or latent processing activity. Because the analysis of SPC gene expression described in this paper indicates that both hPC6 and hPACE4 are present in the furin-defective LoVo cells, the loss of HA processing activity provides convincing evidence that neither PC6 nor PACE4 normally cleave HA, *in vivo*. Therefore, a more cautious interpretation of the HA maturation events would be that at wild-type levels of cellular protease, the endogenous furin is most likely responsible for processing influenza HA *in vivo* (47).

A number of studies have implicated the SPC proteases in HIV-1 gp160 processing to the mature gp120 and gp41 subunits in virally infected human T cells (33, 34, 39, 62–65). An earlier study suggested that hPC1 was capable of processing gp160 *in vitro*, and that the hPC1 gene was expressed in human H9 T cells (39). We were unable to detect expression of a 6.2-kb hPC1 message in either Jurkat, CEM (see Fig. 3*A*, lanes 9–11), or H9 T-cell lines (not shown). We used two different hPC1 probes in our experiments that successfully detected hPC1 by DNA Southern blot analysis (e.g., Fig. 1*A*) and by RNA hybridization of mouse brain, which is known to express PC1 message (Fig. 3*A*, lane 12). The difference in hPC1 expression in T lymphocytes between the two studies may reflect differences in tissue culturing techniques or in the sensitivity of mRNA detection. However, comparative Northern blots of human organs show very limited distribution of hPC1 expression to neuroendocrine cells in brain, pituitary, and pancreas (66), which is not necessarily congruous with hPC1 expression in lymphocytes. Significantly, work from Seidah and colleagues (10) and this study detect no PC1 mRNA in human LoVo cells, which further emphasizes the point that the PC1 protease is not required for gp160 processing (see below).

Several studies have suggested that furin is the host protease responsible for this cleavage activity. The recognition sequence at the cleavage junction (R-E-K-R) between gp120 and gp41 suggests that gp160 is an ideal substrate for furin activity, as shown in studies of furin transfection into a variety of nonlymphoid cell lines, of furin overexpression from vaccinia virus constructs in nonlymphoid and human T-cell lines, and biochemically with purified furin protease (39, 62–64). The furin hypothesis was also attractive because of the relative abundance of furin within the constitutive pathway of a broad spectrum of cells, tissues, and organs. Furthermore, numerous cellular precursors as well as envelope glycoproteins from Newcastle disease virus and influenza virus clearly use furin for processing (44–48, 60, 67–72).

Conflicts with the hypothesis that furin is the host cell protease for HIV-1 gp160 have arisen from several angles. The relative efficiency of HIV-1 gp160 processing in cells transfected with the HIV-1 *env* gene (≈60–80%) was quite high in nonlymphoid cells, such as BSC-1, HeLa, or CV-1 cells and in cells overexpressing the furin protease (2, 62). In contrast, *env* gene transfection into $CD4^-$ or $CD4^+$ lymphocytes results in a lower efficiency (5–20%) of gp160 processing by the endogenous protease, and is identical to that observed in HIV-infected human T cells (1, 15). The results from this work and other studies (39) confirm that furin mRNA is expressed to significant levels in human T cells so that furin expression levels would not explain the lower efficiency of gp160 cleavage in T cells.

Most importantly, LoVo cells encoding a defective form of furin were tested for HIV-1 gp160 processing. LoVo cells transfected with the entire HIV genome exhibit proper gp160 precursor processing and produce infectious virions (47). Another furin-defective cell line, CHO-FD11 fibroblasts, also demonstrated the ability to generate infectious virions (73), reiterating that furin is not required for gp160 maturation. Which of the SPC proteases is then responsible for gp160 cleavage? The hybridization probes used in this study for assessing the cohort of SPC proteases would reveal any member of that family based on their homology with the subtilisin-like catalytic domain, from yeast Kex2 to any of the mammalian enzymes. We cannot eliminate the possibility that another protease outside this family is capable of processing gp160 in T cells, but the weight of evidence supports an SPC protease requirement. Close examination of the SPC expression pattern in LoVo cells reveals the presence of hPC6 gene products in addition to hPACE4 and defective furin messages. By deductive reasoning, since human T cells apparently do not express hPACE4, yet exhibit similar levels of hPC6 gene expression as LoVo cells, we infer that the hPC6 gene products represent the *bona fide* host proteases for HIV-1 gp160 processing.

The presence of multiple SPC enzymes within the cell may mean that alternate SPC proteases serve backup roles to the principal SPC protease for gp160 processing. Precursor processing is nevertheless dependent on the convergence of candidate proteases and their substrates within the secretory pathway. Therefore, because neither Newcastle disease virus nor influenza envelope precursor proteins were cleaved in LoVo cells (46, 47), the multiple protease hypothesis seems unlikely to explain gp160 processing activity in LoVo cells. Furthermore, the presence of furin in human T cells raises important questions about the routing of gp160 in the constitutive versus regulated secretory pathways. We are directly testing whether or which of the two hPC6 protease isoforms that we have cloned is responsible for the gp160 processing activity in human $CD4^+$ T lymphocytes, and which endogenous precursor polypeptides require PC6 activity in human T cells, *in vivo*. Elucidation of these issues is critical. Characterization of the biochemical properties of the cellular gp160 processing protease can be used in the design of specific SPC protease inhibitors capable of interfering with HIV infectivity and AIDS.

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1. Willey, R. L., Bonifacino, J. S., Potts, B. J., Martin, M. A. & Klausner, R. D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9580–9584.
2. Earl, P. L., Moss, B. & Doms, R. W. (1991) *J. Virol.* **65,** 2047–2055.
3. Haseltine, W. (1991) *FASEB J.* **5,** 2349–2360.
4. Lifson, J. D., Feinberg, M. B., Reyes, G. R., Rabin, L., Banapour, B., Chakrabarti, S., Moss, B., Wong-Staal, F., Steimer, K. S. & Engleman, E. G. (1986) *Nature (London)* **323,** 725–728.
5. Sodroski, J., Goh, W. C., Rosen, C., Campbell, K. & Haseltine, W. A. (1986) *Nature (London)* **322,** 470–474.
6. Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W. C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W. & Sodroski, J. (1987) *Science* **237,** 1351–1355.
7. Landau, N. R., Warton, M. & Littman, D. R. (1988) *Nature (London)* **334,** 159–162.
8. Barr, P. (1991) *Cell* **66,** 1–3.
9. Steiner, D. F., Smeekens, S. P., Ohagi, S. & Chan, S. J. (1992) *J. Biol. Chem.* **267,** 23435–23438.
10. Seidah, N. G., Chretien, M. & Day, R. (1994) *Biochimie* **76,** 197–209.
11. Walker, B. D., Kowalski, M., Goh, W. C., Kozarsky, K., Krieger, M., Rosen, C., Rohrschneider, L., Haseltine, W. & Sodroski, J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8120–8124.
12. McCune, J. M., Rabin, L. B., Feinberg, M. B., Lieberman, M., Kosek, J. C., Reyes, G. R. & Weissman, I. L. (1988) *Cell* **53,** 55–67.
13. Freed, E. O., Myers, D. J. & Risser, R. (1989) *J. Virol.* **63,** 4670–4675.
14. Bosch, V. & Pawlita, M. (1990) *J. Virol.* **64,** 2337–2344.
15. Earl, P. L., Koenig, S. & Moss, B. (1991) *J. Virol.* **65,** 31–41.
16. Julius, D., Brake, A., Blair, L., Kunisawa, R. & Thorner, J. (1984) *Cell* **37,** 1075–1089.

17. Fuller, R. S., Brake, A. J. & Thorner, J. (1989) *Science* **246,** 482–486.
18. Mizuno, K., Nakamura, T. & Matsuo, H. (1989) *Biochem. Biophys. Res. Commun.* **164,** 780–787.
19. Hatsuzawa, K., Hosaka, M., Nakagawa, T., Nagase, M., Shoda, A., Murakami, K. & Nakayama, K. (1990) *J. Biol. Chem.* **265,** 22075–22078.
20. Barr, P. J., Mason, O. B., Landsberg, K. E., Wong, P. A., Kiefer, M. C. & Brake, A. J. (1991) *DNA Cell Biol.* **10,** 319–328.
21. Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M. & Chretien, M. (1990) *DNA Cell Biol.* **9,** 415–424.
22. Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M. & Chrétien, M. (1991) *Mol. Endocrinol.* **5,** 111–122.
23. Smeekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J. & Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 340–344.
24. Smeekens, S. P. & Steiner, D. F. (1990) *J. Biol. Chem.* **265,** 2997–3000.
25. Nakayama, K., Kim, W.-S., Torij, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T. & Murakami, K. (1992) *J. Biol. Chem.* **267,** 5897–5900.
26. Seidah, N. G., Day, R., Hamelin, J., Gaspar, A., Collard, M. W. & Chretien, M. (1992) *Mol. Endocrinol.* **6,** 1559–1570.
27. Kiefer, M. C., Tucker, J. E., Joh, R., Landsberg, K. E., Saltman, D. & Barr, P. J. (1991) *DNA Cell Biol.* **10,** 757–769.
28. Hosaka, M., Murakami, K. & Nakayama, K. (1994) *Biomed. Res.* **15,** 383–390.
29. Lusson, J., Vieau, D., Hamelin, J., Day, R., Chrétien, M. & Seidah, N. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6691–6695.
30. Nakagawa, T., Hosaka, M., Torii, S., Watanabe, T., Murakami, K. & Nakayama, K. (1993) *J. Biochem.* **113,** 132–135.
31. Nakagawa, T., Murakami, K. & Nakayama, K. (1993) *FEBS Lett.* **327,** 165–171.
32. Tsuji, A., Hine, C., Mori, K., Tamai, Y., Higashine, K., Nagamune, H. & Matsuda, Y. (1994) *Biochem. Biophys. Res. Commun.* **202,** 1452–1459.
33. Franzusoff, A., Volpe, A. M., Josse, D., Pichuantes, S. & Wolf, J. R. (1995) *J. Biol. Chem.* **270,** 3154–3159.
34. Moulard, M., Achstetter, T., Kieny, M.-P., Montagnier, L. & Bahraoui, E. (1994) *Eur. J. Biochem.* **225,** 565–572.
35. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
36. Barnes, W. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2216–2220.
37. Horton, R. M., Cai, C., Ho, S. N. & Pease, L. R. (1990) *Biotechniques* **8,** 528–535.
38. Hwang, S. S., Boyle, T. J., Lyerly, H. K. & Cullen, B. R. (1991) *Science* **253,** 71–74.
39. Decroly, E., Vandenbranden, M., Ruysschaert, J.-M., Cogniaux, J., Jacob, G. S., Howard, S. C., Marshall, G., Kompelli, A., Basak, A., Jean, F., Lazure, C., Benjannet, S., Chretien, M., Day, R. & Seidah, N. G. (1994) *J. Biol. Chem.* **269,** 12240–12247.
40. Siezen, R. J., Creemers, J. W. M. & Van de Ven, W. J. M. (1994) *Eur. J. Biochem.* **222,** 255–266.
41. Mizuno, K., Nakamura, T., Ohshima, T., Tanaka, S. & Matsuo, H. (1988) *Biochem. Biophys. Res. Commun.* **156,** 246–254.
42. Drewinko, B., Romsdahl, M. M., Yang, L. Y., Ahearn, M. J. & Trujillo, J. M. (1976) *Cancer Res.* **36,** 467–475.
43. Takahashi, S., Kasai, K., Hatsuzawa, K., Kitamura, N., Misumi, Y., Ikehara, Y., Murakami, K. & Nakayama, K. (1993) *Biochem. Biophys. Res. Commun.* **195,** 1019–1026.
44. Komada, M., Hatsuzawa, K., Shibamoto, S., Ito, F., Nakayama, K. & Kitamura, N. (1993) *FEBS Lett.* **328,** 25–29.
45. Tsuneoka, M., Nakayama, K., Hatsuzawa, K., Komada, M., Kitamura, N. & Mekada, E. (1993) *J. Biol. Chem.* **268,** 26461–26465.
46. Horimoto, T., Nakayama, K., Smeekens, S. & Kawaoka, Y. (1994) *J. Virol.* **68,** 6074–6078.
47. Ohnishi, Y., Shioda, T., Nakayama, K., Iwata, S., Gotoh, B., Hamaguchi, M. & Nagai, Y. (1994) *J. Virol.* **68,** 4075–4079.
48. Dubois, C. M., Laprise, M. H., Blanchette, F., Gentry, L. E. & Leduc, R. (1995) *J. Biol. Chem.* **270,** 10618–10624.
49. Creemers, J. W. M., Vey, M., Shäfer, W., Ayoubi, T. A. Y., Roebroek, A. J. M., Klenk, H. D., Garten, W. & Van de Ven, W. J. M. (1995) *J. Biol. Chem.* **270,** 2695–2702.
50. Leduc, R., Molloy, S. S., Thorne, B. A. & Thomas, G. (1992) *J. Biol. Chem.* **267,** 14304–14308.
51. Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E. & Thomas, G. (1994) *EMBO J.* **13,** 18–33.
52. Vey, M., Schäfer, W., Berghöfer, S., Klenk, H.-D. & Garten, W. (1994) *J. Cell Biol.* **127,** 1829–1842.
53. Zhou, Y. & Lindberg, I. (1994) *J. Biol. Chem.* **269,** 18408–18413.
54. Braks, A. M. & Martens, G. J. M. (1994) *Cell* **78,** 263–273.
55. Zhu, X. & Lindberg, I. (1995) *J. Cell Biol.* **129,** 1641–1650.
56. Shennan, K. I. J., Taylor, N. A., Jermany, J. L., Matthews, G. & Docherty, K. (1995) *J. Biol. Chem.* **270,** 1402–1407.
57. Hosaka, M., Nagahama, M., Kim, W.-S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. & Nakayama, K. (1991) *J. Biol. Chem.* **266,** 12127–12130.
58. Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R. & Thomas, G. (1992) *J. Biol. Chem.* **267,** 16396–16402.
59. Takahashi, S., Hatsuzawa, K., Watanabe, T., Murakami, K. & Nakayama, K. (1994) *J. Biochem.* **116,** 47–52.
60. Stieneke-Grober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H.-D. & Garten, W. (1992) *EMBO J.* **11,** 2407–2414.
61. Walker, J. A., Sakaguchi, T., Matsuda, Y., Yoshida, T. & Kawaoka, Y. (1992) *Virology* **190,** 278–287.
62. Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H.-D. & Garten, W. (1992) *Nature (London)* **360,** 358–361.
63. Anderson, E. D., Thomas, L., Hayflick, J. S. & Thomas, G. (1993) *J. Biol. Chem.* **263,** 24887–24891.
64. Morikawa, Y., Barsow, E. & Jones, I. (1993) *J. Virol.* **67,** 3601–3604.
65. Moulard, M., Montagnier, L. & Bahraoui, E. (1994) *FEBS Lett.* **338,** 281–284.
66. Seidah, N. G., Hamelin, J., Gaspar, A. M., Day, R. & Chrétien, M. (1992) *DNA Cell Biol.* **11,** 283–289.
67. Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J. & Thomas, G. (1990) *J. Cell Biol.* **111,** 2851–2859.
68. Moehring, J. M., Inocencio, N. M., Robertson, B. J. & Moehring, T. J. (1993) *J. Biol. Chem.* **268,** 2590–2594.
69. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A. & Fuller, R. S. (1994) *J. Biol. Chem.* **269,** 25830–25837.
70. Sakaguchi, T., Fujii, Y., Kiyotani, K. & Yoshida, T. (1994) *J. Gen. Virol.* **75,** 2821–2827.
71. Hendy, G. N., Bennett, H. P. J., Gibbs, B. F., Lazure, C., Day, R. & Seidah, N. G. (1995) *J. Biol. Chem.* **270,** 9517–9525.
72. Pei, D. & Weiss, S. J. (1995) *Nature (London)* **375,** 244–247.
73. Gu, M., Rappaport, J. & Leppla, S. H. (1995) *FEBS Lett.* **365,** 95–97.